Effects of repetitive tetanic stimulation at long intervals on excitation–contraction coupling in frog skeletal muscle

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1. Single skeletal muscle fibres of *Xenopus* frogs were used to investigate the possibility that excitation–contraction (E–C) coupling can be impaired under conditions of elevated intracellular free Ca^{2+} ($[Ca^{2+}]_i$).

2. Fibres were stimulated with a train of up to 200 tetani at 10 or 20 s intervals; this long-interval stimulation (LIS) scheme was chosen to minimize fatigue. After LIS, fibres were exposed to hypotonic Ringer solution for 5 min. At the end of LIS, force was about 90% of the original and the hypotonic challenge did not result in any force depression.

3. Caffeine, terbutaline and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone increased both basal and tetanic $[Ca^{2+}]_i$. In ten out of thirteen fibres, the presence of any of these drugs during LIS resulted in a force reduction to about 10% of the control when fibres were returned to normal Ringer solution after the hypotonic challenge. Force production was severely depressed for at least 20 min and then recovered to control levels within 120 min.

4. Neither protease inhibitors nor a scavenger of reactive oxygen species prevented the impairment of E–C coupling.

5. It is concluded that after a period of elevated $[Ca^{2+}]_i$ E–C coupling in frog skeletal muscle becomes sensitive to the mechanical stress induced by exposure to hypotonic solution. The underlying molecular basis for this remains unclear.

Following fatigue, muscle performance is often impaired for many hours. This manifests itself as a reduced force response to low frequency stimulation in mammalian muscle (Jones, Howell, Roussos & Edwards, 1982; Westerblad, Duty & Allen, 1993) or a decreased tetanic force production in frog muscle (Westerblad & Lännergren, 1986). The underlying mechanism appears to be the same in both cases, impaired excitation–contraction (E–C) coupling.

In frog skeletal muscle fibres, the extent and duration of reduced performance can be greatly increased if a fibre is exposed to a moderately hypotonic Ringer solution for 5 min immediately after the induction of fatigue (Bruton, Lännergren & Westerblad, 1995). During the period of depressed force, electrical stimulation results in normal action potentials and application of a high concentration of caffeine (12 mM) gives high force contractures. We therefore concluded that the long-lasting loss of force production, induced by fibre swelling and subsequent shrinkage due to the hypotonic treatment, reflects a weakening of a mechano-sensitive link involved in E–C coupling (Bruton *et al.* 1995). It has been shown that E–C coupling in skinned toad muscle fibres is disrupted when Ca^{2+} is elevated to high levels (23 μM; Lamb, Junankar & Stephenson, 1995). Thus the failure of E–C coupling that is seen after fatigue and which is exaggerated by hypotonic treatment might be caused by the repeated pulses of high Ca^{2+} which occur during the induction of fatigue. The mechanism behind this might, for example, be activation of Ca^{2+}-dependent lipases or proteases (Armstrong, 1990; Belcastro, 1993), which in turn modify or damage structures critical to E–C coupling.

During fatigue, there are both metabolic changes and repeated pulses of Ca^{2+}. To minimize metabolic changes and to look at the effects of Ca^{2+} only, we have used a protocol with tetanic stimulation at long (10–20 s) intervals (long-interval stimulation, LIS). The force decline was small during LIS and there was no additional force decline when fibres were exposed to hypotonic solution after LIS. However, there is an increase in the basal free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) throughout fatigue and also an increase in tetanic $[Ca^{2+}]_i$ in early fatigue (Lee, Westerblad & Allen, 1991). In order to mimic this, LIS was also performed in the presence of drugs which have been shown to elevate $[Ca^{2+}]_i$: caffeine, terbutaline or 2,5-di(*tert*-butyl)-benzohydroquinone (TBQ or tBuHQ). When LIS was performed in the

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presence of any of these drugs, exposure to the hypotonic solution resulted in a marked force depression in the vast majority of experiments. Thus repeated pulses of high $[Ca^{2+}]_i$ may weaken E–C coupling.

METHODS

General

Xenopus laevis frogs were stunned, beheaded and pithed. Single muscle fibres were isolated from the lumbrical muscles. Fibres were mounted in a small chamber and their length adjusted so that isometric tetanic force was maximal. Supramaximal tetanic stimulation (70 Hz, 350–400 ms) was applied by platinum electrodes lying parallel to the long axis of the muscle fibre. Force was detected by an Akers AE 801 force transducer (SensoNor, Horten, Norway), amplified and recorded on a pen recorder and personal computer. Tetanic force was measured as peak force and is expressed as a percentage of the force in tetani produced under control conditions. All values are expressed as means ± S.E.M., and Student's paired *t* test was used to test for statistical significance; the significance level was set at 0·05 throughout.

Solutions and drugs

Fibres were superfused with a Ringer solution containing (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; sodium phosphate, 3·0; EDTA, 0·1 (pH 7·0). All experiments were carried out at room temperature (22 °C). In order to increase $[Ca^{2+}]_i$, one of the following drugs was added to the Ringer solution: caffeine (0·5 mM), terbutaline (10 μM; Astra-Draco, Lund, Sweden) or 2,5-di(*tert*-butyl)-benzohydroquinone (TBQ or tBuHQ; 0·1 μM; Aldrich-Chemie). Fibres were bathed for 3 min in caffeine, 10 min in terbutaline and 15 min in TBQ before the experiments started; during these periods no stimulations were given. All three drugs were present throughout LIS.

In some experiments, fibres were exposed to a protease inhibitor cocktail consisting of E64-d (15 μM; Taisho Pharmaceutical, Osaka, Japan), pepstatin (50 μM; Sigma), leupeptin (100 μM; Sigma) and phenylmethylsulphonyl fluoride (PMSF; 100 μM; Sigma). *N*-acetylcysteine (NAC; Sigma) was used as a scavenger of reactive oxygen species (free radicals) at concentrations of 0·1 to 1 mM; higher concentrations blocked the contractile response to electrical stimulation. Both the protease inhibitor cocktail and NAC were applied 15 min prior to LIS and were then present during LIS and during the 5 min period in hypotonic solution.

Long-interval stimulation protocols

The term long-interval stimulation (LIS) is used to describe a train of tetani with intervals of 10 or 20 s between tetani depending on whether type 2 (medium-oxidative) or type 1 (low-oxidative) muscle fibres were used. Fibres were typed according to their microscopical appearance (type 1 fibres are somewhat larger and more transparent than type 2 fibres) and isometric contractile properties (type 1 fibres have a shorter contraction time and a higher twitch/tetanus ratio than type 2 fibres) (e.g. Lännergren, Lindblom & Johansson, 1982). The choice of interval between tetani was based on previous work which predicted that such intervals were sufficient for ATP production to match ATP usage in the two muscle fibre types, respectively (van der Laarse, Elzinga & Woledge, 1989). The number of tetani in a train was typically 100 for type 1 fibres and 200 for type 2 fibres. At the end of LIS, fibres

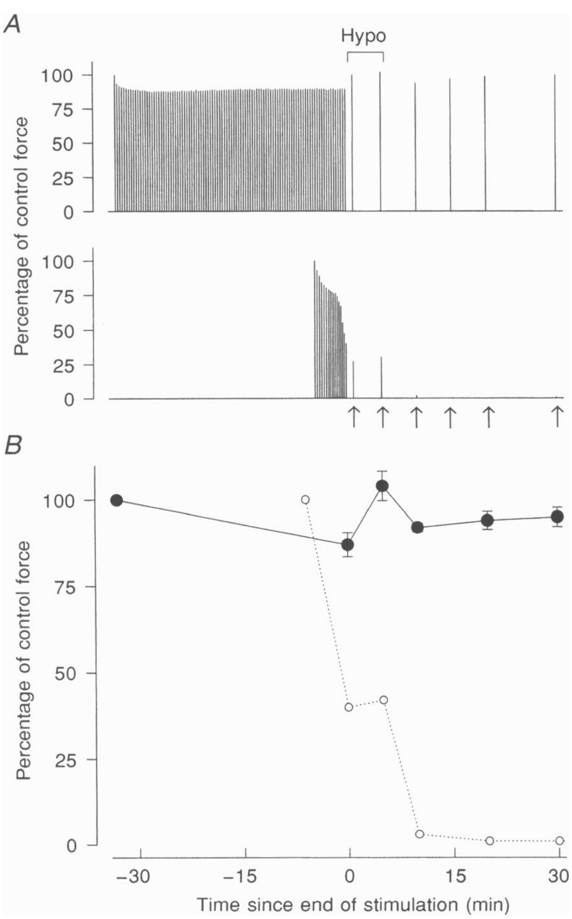

Figure 1. Force is not abolished in fibres subjected to long-interval stimulation followed by 5 min exposure to 80% NaCl Ringer solution

A, upper trace shows tetanic force responses in a fibre subjected to LIS (100 tetani given at 20 s intervals) followed by exposure to hypotonic Ringer solution for 5 min. Lower trace shows tetanic force responses from another fibre which was fatigued (81 tetani at 4 and 3 s intervals; only every fifth tetanus is shown) and then exposed to hypotonic Ringer solution. Arrows below the lower trace indicate times of stimulation. Note that after hypotonic exposure force is abolished in the fatigued fibre while there is no obvious force decline in the fibre subjected to LIS. *B*, mean force ± S.E.M. ($n = 4$) from fibres subjected to LIS and then exposed to 80% NaCl Ringer solution (filled symbols and continuous line). For comparison, the dotted line and open symbols indicate mean values for 9 fibres fatigued to 40% of initial force and then exposed to the hypotonic solution (data taken from Bruton *et al.* 1995). The bracket indicating the period of hypotonic exposure (Hypo) in *A* and the time axis in *B* apply to both *A* and *B*.

were exposed for 5 min to a Ringer solution containing 80% of the usual NaCl concentration and then returned to normal Ringer solution.

Measurement of $[Ca^{2+}]_i$

Fibres were stimulated with a train of five tetani at 10 s intervals and the myoplasmic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured with the fluorescent Ca^{2+} indicator indo-1, which was pressure-injected into the fibre. The methods for measuring the fluorescence signals and converting these into $[Ca^{2+}]_i$ have been described previously (Westerblad & Allen, 1996). Basal $[Ca^{2+}]_i$ was measured during the 100 ms prior to electrical stimulation and tetanic $[Ca^{2+}]_i$ during the last 100 ms of the tetanus plateau.

RESULTS

Tetanic force after LIS and hypotonic challenge and comparison with fatigue

The upper trace of Fig. 1*A* shows the typical response of a fibre subjected to LIS followed by exposure to hypotonic Ringer solution for 5 min. During LIS force fell by about 10%. Force then increased somewhat in the hypotonic solution and thereafter remained close to the control force. For comparison, the lower trace of Fig. 1*A* shows a fibre which was fatigued to 40% of the original force by repeated tetani at 4 and 3 s intervals (only every fifth tetanus is shown) and then exposed to the hypotonic solution. In this situation, force was completely abolished after the period in hypotonic solution and showed no recovery during 30 min.

Figure 1*B* summarizes the mean response of four fibres (two type 1 and two type 2 fibres) subjected to LIS and hypotonic Ringer solution. At the end of LIS, force was $87 \pm 4\%$ of the initial value; it recovered rapidly and was $95 \pm 3\%$ at 30 min, when the period of observation was ended. In contrast, fatigued fibres exposed to hypotonic solution had a completely different response, with essentially no response to tetanic stimulation at 30 min, and even at 120 min, force had recovered to only about 10% of the control (data from Bruton *et al.* 1995). These results show that when a number of tetani (similar to that needed to produce fatigue) are given at 10 or 20 s intervals, E–C coupling is not impaired.

Drug-induced changes in $[Ca^{2+}]_i$

In order to increase the $[Ca^{2+}]_i$ load during LIS, we used three drugs (caffeine, terbutaline and TBQ) known to

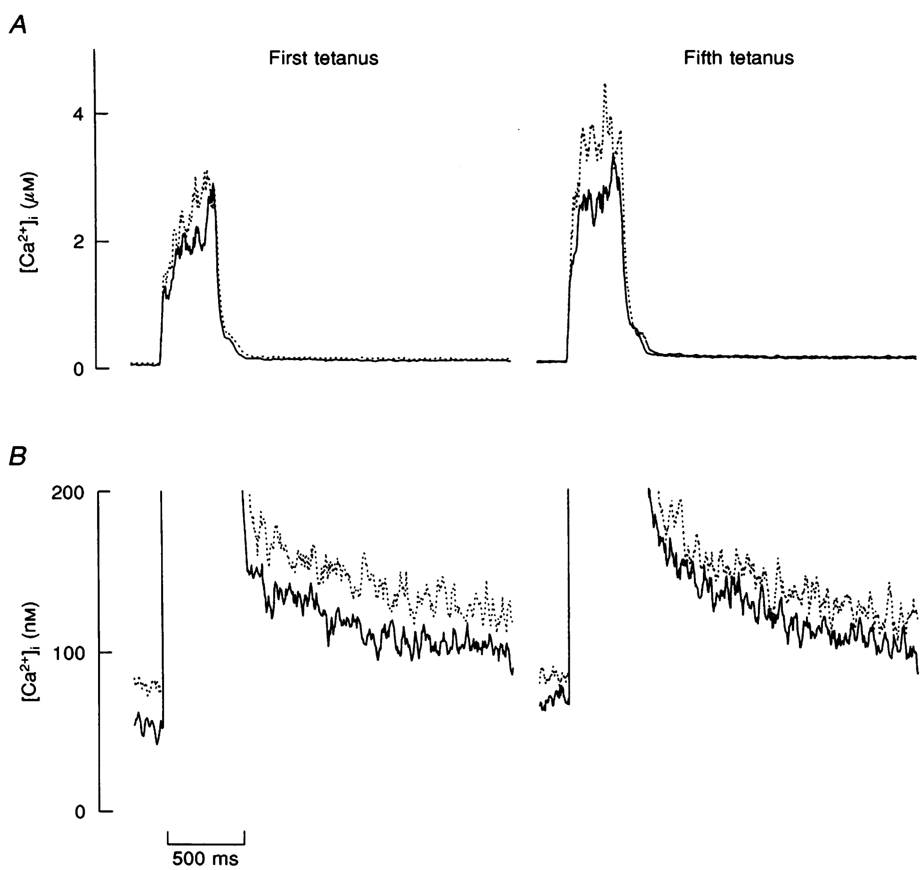

Figure 2. Both basal and tetanic $[Ca^{2+}]_i$ are higher in the presence than in the absence of caffeine

Typical $[Ca^{2+}]_i$ records from 1 fibre during the first and fifth tetanus of a series of 5 tetani at 10 s intervals. Full records are shown in *A*; *B* shows the same records on an expanded scale so that changes in basal $[Ca^{2+}]_i$ become visible. Continuous lines indicate records obtained in normal Ringer solution and dotted lines those obtained in the presence of 0·5 mM caffeine. Note that both tetanic and basal Ca^{2+} levels are higher in the presence of caffeine than in normal Ringer solution. The time scale applies to all records.

increase $[Ca^{2+}]_i$. To verify that the drugs increased $[Ca^{2+}]_i$ in the present preparation also, we compared $[Ca^{2+}]_i$ during a series of five tetani given at 10 s intervals in normal Ringer solution and after addition of any of the drugs. Figure 2 shows a typical record of $[Ca^{2+}]_i$ at the start and end of this series in the absence and presence of caffeine. It can be seen that both basal and tetanic $[Ca^{2+}]_i$ were higher in the presence of caffeine. Similar results were obtained with terbutaline and TBQ; since there was no significant difference between the ability of the three drugs to increase $[Ca^{2+}]_i$, we have pooled $[Ca^{2+}]_i$ data obtained with all three drugs in the following.

In normal Ringer solution with no drugs added, basal $[Ca^{2+}]_i$ measured in four fibres was 74·0 ± 14·0 nM at the start and 79·6 ± 13·3 nM at the end of the series of five tetani. Tetanic $[Ca^{2+}]_i$ was 2·16 ± 0·42 and 2·29 ± 0·41 μM during the final 100 ms of the first and fifth tetanus, respectively. Figure 3 shows pooled data from twelve experiments (four with each drug) and compares $[Ca^{2+}]_i$ with and without drugs present. In the presence of drugs, both basal and tetanic $[Ca^{2+}]_i$ were significantly elevated in the first tetanus and increased further during the series of tetani. Thus, during a train of tetani, the presence of caffeine, terbutaline and TBQ will result in higher $[Ca^{2+}]_i$.

LIS and hypotonic challenge in the presence of drugs that elevate $[Ca^{2+}]_i$

The typical responses of three different fibres subjected to LIS and exposure to hypotonic Ringer solution in the presence of terbutaline (*A*), caffeine (*B*) and TBQ (*C*) are shown in Fig. 4. Following the 5 min exposure to hypotonic Ringer solution, force fell to a very low level in all three fibres. This marked force depression persisted for at least 20 min and at 80 min peak force was still markedly smaller than in control, and sag (rapid decline of force during the tetanus) was present in the fibres exposed to terbutaline and TBQ.

By the end of LIS, force was 90 ± 11% ($n = 3$), 90 ± 7% ($n = 5$) and 88 ± 1% ($n = 5$) in terbutaline, caffeine and TBQ, respectively. During the 5 min period in hypotonic solution force remained high in all fibres. However, on return to the normal Ringer solution ten of the thirteen fibres showed a marked force depression and the mean minimum force in these fibres was 13 ± 8% of the control. Force recovered to 80 ± 6% by 80 min, but a marked sag was nearly always present at this time; within 120 min force had recovered to 90% and sag was less prominent. In the remaining three fibres (two fibres exposed to caffeine and one fibre to terbutaline), there was no significant fall in force as a result of LIS followed by hypotonic treatment; the minimum force in these three fibres was 94 ± 7%. These experiments show that LIS in the presence of drugs which increase $[Ca^{2+}]_i$ may severely weaken E–C coupling. However, a minority of the fibres showed no signs of a weakened E–C coupling, thus behaving as fibres given LIS in the absence of $[Ca^{2+}]_i$-elevating drugs.

There was no obvious difference between type 1 and type 2 fibres concerning their response to LIS in the presence of $[Ca^{2+}]_i$-elevating drugs. Of the thirteen fibres tested with

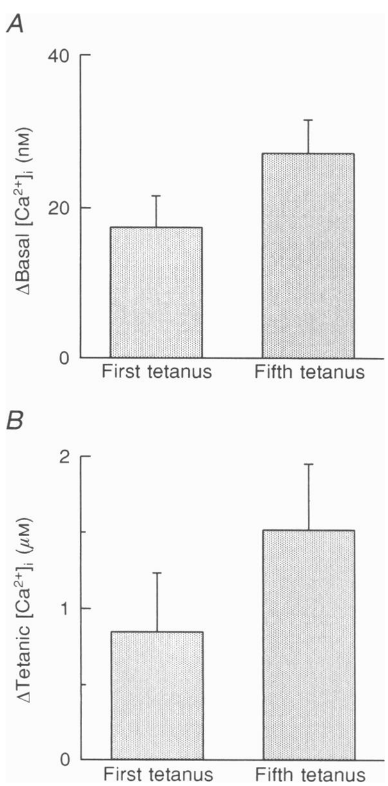

Figure 3. Terbutaline, caffeine and TBQ increase both basal and tetanic $[Ca^{2+}]_i$

Pooled data of the increases in basal (*A*) and tetanic (*B*) $[Ca^{2+}]_i$ from 12 experiments (4 experiments with each drug). Measurements were performed during the first and last tetanus of a series of 5 tetani at 10 s intervals. Basal $[Ca^{2+}]_i$ is the mean value obtained during the last 100 ms before the first and fifth tetanus; tetanic $[Ca^{2+}]_i$ refers to the mean during the last 100 ms of electrical stimulation in the same tetani. Values are means ± S.E.M.; all increases are significant ($P < 0.05$).

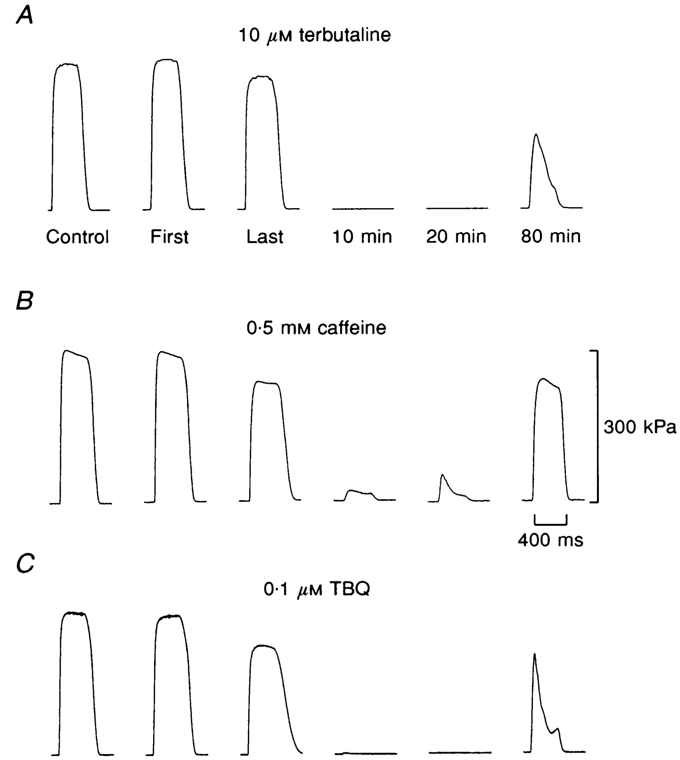

Figure 4. Long-interval stimulation and exposure to 80% NaCl Ringer solution greatly reduces force in fibres exposed to drugs which raise $[Ca^{2+}]_i$

Typical force records from 3 different fibres equilibrated in 10 μM terbutaline (*A*), 0·5 mM caffeine (*B*) or 0·1 μM TBQ (*C*) are shown. Control is the force obtained in normal Ringer solution. First and Last refer to the force at the start and end of a train of 200 tetani in the presence of the drug and 10, 20 and 80 min indicate the time since the end of the train. Force and time calibrations shown to the right apply to all traces.

the drugs, six were type 1 and seven type 2 and of the fibres which showed no significant fall of force, one was type 1 and two type 2.

Are proteases or free radicals involved in weakening of E–C coupling?

Lastly, we looked at what processes might be involved in mediating the action of elevated $[Ca^{2+}]_i$. In order to examine the involvement of proteases, fibres were exposed to a protease inhibitor cocktail (see Methods). This protease inhibitor cocktail did not prevent the marked depression of force and did not accelerate recovery of force (Fig. 5*A*). A similar lack of protection was seen in a further two fibres tested. Thus, activation of proteases does not seem to play a role in the abolition of force.

Figure 5*B* shows an experiment in the presence of NAC (a general scavenger of reactive oxygen species; Halliwell, 1991). The drug did not prevent the loss of force and did not enhance recovery in this fibre and in two other fibres tested. This suggests that reactive oxygen species play little role in the observed effect.

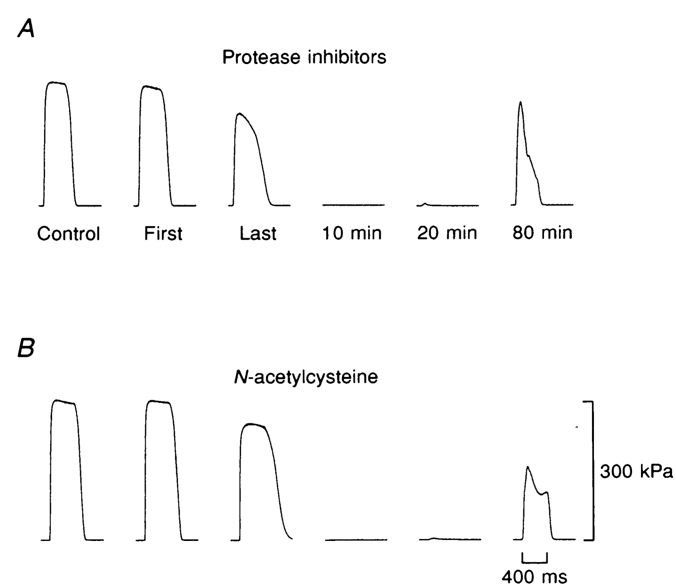

Figure 5. Neither protease inhibitors nor a free radical scavenger prevent the adverse effects of long-interval stimulation and exposure to hypotonic Ringer solution

Typical records from 2 different fibres subjected to LIS and hypotonic exposure in the presence of 0·1 μM TBQ and exposure to a protease inhibitor cocktail (*A*) or 1 mM NAC (*B*). Control is tetanic force obtained in normal solution. First and Last are the force at the start and end, respectively, of a train of 200 tetani in the presence of the drug and 10, 20 and 80 min indicate the time since the end of the train. Force and time calibrations shown to the right apply to all traces.

DISCUSSION

Metabolic changes *vs.* increased $[Ca^{2+}]_i$ as possible mechanisms underlying the force depression

In a previous study (Bruton *et al.* 1995), we observed an almost complete and long-lasting force depression when fatigued fibres were returned to normal Ringer solution after being exposed for 5 min to a Ringer solution in which the NaCl concentration was reduced to 80% of normal. This hypotonic challenge is rather modest and would increase the fibre cross-sectional area by about 15% (Blinks, 1965). During the period of force depression, the force production during caffeine contractures was normal and normal action potentials with clear negative after-potentials could be elicited, which indicates that the t-tubules are intact and propagate action potentials (Gage & Eisenberg, 1969). Thus we concluded that the force depression was caused by failing E–C coupling. The mechanism underlying this E–C coupling failure was unclear and the present study was aimed at distinguishing between two main possibilities: metabolic changes related to fatigue and increased $[Ca^{2+}]_i$. The LIS protocol was designed so that changes due to increased energy metabolism should be minimal. During LIS force normally fell by only 10%, while the force decline in fatigue was 60% (Bruton *et al.* 1995). Since the force decline in fatigue is most probably caused by metabolic changes (e.g. Allen, Lännergren & Westerblad, 1995), it seems clear that the metabolic changes which occurred during LIS were much smaller than those in fatigue. This would indicate that the fibre swelling occurring during LIS is smaller than that in fatigue, because this swelling is most probably caused by an increased number of osmotically active particles originating from increased metabolic activity (e.g. Lännergren, 1990).

LIS followed by exposure to hypotonic Ringer solution had no significant effect on subsequent force production in normal Ringer solution. On the other hand, on return to normal Ringer solution after hypotonic challenge following LIS in the presence of drugs which elevate $[Ca^{2+}]_i$, force became severely depressed in the vast majority of fibres. This shows that a period with repeated Ca^{2+} pulses can make the E–C coupling susceptible to hypotonic treatment. However, even with the $[Ca^{2+}]_i$-elevating drugs present, some fibres showed no significant force depression. Thus, this force depression seems to be an all-or-none phenomenon: either there is a very marked force depression or there is no depression at all.

It may be argued that the increased sensitivity to hypotonic treatment after LIS observed with the $[Ca^{2+}]_i$-elevating drugs is a side effect of the drugs rather than a specific Ca^{2+} effect. However, this seems unlikely since the present drugs are not structurally related and employ different intracellular systems to exert their actions. Terbutaline is a β-adrenergic agonist, which exerts its effects via cAMP-dependent phosphorylation of proteins and may elevate $[Ca^{2+}]_i$ by acting on the sarcoplasmic reticulum (SR) Ca^{2+}-release mechanism (Cairns, Westerblad & Allen, 1993) and/or by increasing the influx of extracellular Ca^{2+} (Williams & Barnes, 1989). Caffeine is a methylxanthine, which may increase $[Ca^{2+}]_i$ by directly binding to and activating the SR Ca^{2+}-release channels (Rousseau, LaDine, Liu & Meissner, 1988) and by inhibiting the SR Ca^{2+} pumps (Fryer & Neering, 1989; Allen & Westerblad, 1995). Finally, the hydroquinone TBQ seems to be a specific inhibitor of the SR Ca^{2+} pumps and elevates $[Ca^{2+}]_i$ by reducing the rate of SR Ca^{2+} uptake (Westerblad & Allen, 1994). Thus, the only property these drugs obviously share is that they all elevate $[Ca^{2+}]_i$.

There are two important differences between the force depression caused by hypotonic treatment in fatigue and after LIS in the presence of $[Ca^{2+}]_i$-elevating drugs. First, all fibres exposed to hypotonic Ringer solution in fatigue displayed a marked force depression (Bruton *et al.* 1995), whereas a minority of the fibres subjected to LIS and $[Ca^{2+}]_i$-elevating drugs showed no significant force depression. Second, recovery was much slower in fatigued fibres than in the LIS fibres which showed force depression: recovery to 90% force took about 17 h in fatigued fibres and occurred within 2 h in LIS fibres. Thus, the deleterious effect of exposure to hypotonic Ringer solution was much more prominent in fatigued fibres. This might be explained by a larger Ca^{2+} load during fatigue; for example, the increase in basal $[Ca^{2+}]_i$ obtained with the $[Ca^{2+}]_i$-elevating drugs was less than 40 nM (Fig. 3), while the basal $[Ca^{2+}]_i$ in fatigue is increased by about 300 nM (Lee *et al.* 1991). Alternatively, there may be some undefined factor in fatigue that makes fatigued fibres more vulnerable. Recent studies support the increased Ca^{2+} load explanation: the extent of Ca^{2+}-induced E–C uncoupling depends upon both the duration of exposure and the level of Ca^{2+} (Lamb *et al.* 1995); in intact mouse muscle fibres elevated $[Ca^{2+}]_i$ reduces subsequent tetanic $[Ca^{2+}]_i$ and force, especially at low stimulation frequency, which indicates impaired E–C coupling (Chin & Allen, 1996).

Possible mechanisms underlying Ca^{2+}-induced failure of E–C coupling

The precise mechanisms involved in the action of Ca^{2+} are unclear. No recordings of action potentials were performed during the period of force depression after LIS and therefore an effect on action potential propagation into the t-tubules cannot be excluded. However, normal action potentials were recorded during force depression after fatigue (Bruton *et al.* 1995), which most probably caused more severe fibre swelling than did LIS and hence an increased risk of action potential propagation failure in the t-tubules (cf. Dulhunty & Gage, 1973).

The transient nature of the loss of force-generating capacity suggests that the action of Ca^{2+} is discrete and does not involve generalized damage to some cellular structure. In the present experiments, there was no evidence that proteases were involved, as the combination of the protease inhibitors E64-d, leupeptin, pepstatin and PMSF (in concentrations sufficient to block proteolysis) had no protective effect. Furthermore, force recovery occurred within 2 h, which is too fast for any important protein resynthesis to take place. This is in agreement with our previous study on fatigued fibres, in which each of these protease inhibitors was found to be ineffectual (Bruton *et al.* 1995).

Free oxygen radicals are known to be produced at an increased rate in exercising muscle (Reid, Haack, Franchek, Valberg, Kobzik & West, 1992) and these can damage or modify the structure of proteins (Neuzil, Gebicki & Stocker, 1993). However, in the present experiments, NAC, which is a potent free radical scavenger (Halliwell, 1991), did not prevent the loss of force. Furthermore, dimethyl sulphoxide, another free radical scavenger, could not prevent the E–C uncoupling in fatigued fibres exposed to hypotonic Ringer solution (Bruton *et al.* 1995). Thus, it seems doubtful that free radicals are involved in the E–C uncoupling.

Ca^{2+}-activated lipases may be involved in the observed E–C uncoupling. We recently attempted to test this by applying lipase inhibitors, but the drugs used (bromophenylacylbromide and nordihydroguairetic acid) made fibres unable to respond to electrical stimulation and caused depolarization (Bruton, Westerblad & Lännergren, 1994). Thus the hypothesis could not be put to the test and hence an involvement of Ca^{2+}-activated lipases in the E–C uncoupling cannot be ruled out.

Another possible cause of the E–C uncoupling is phosphorylation of proteins, which is known to alter their quaternary structure and function and which is often induced by activation of Ca^{2+}–calmodulin-dependent protein kinases (e.g. Meissner, 1994). Finally, direct binding of Ca^{2+} to a site on the dihydropyridine or ryanodine receptors or some other critical site in E–C coupling cannot be ruled out, because Ca^{2+} is well known to cause conformational changes in many intracellular proteins (Williams, 1986).

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